

Optimizing SSR-PCR system of *Panax ginseng* by orthogonal design

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Abstract: An orthogonal design was used to optimize SSR-PCR amplification system using *Panax ginseng* genomic DNA as template. Four levels of five factors (DNA template, *Taq* DNA polymerase, Mg^{2+} , primer, and dNTP) and annealing temperature have been tested separately in this system. The results demonstrated the reaction efficiency was affected by these factors. Based on the results, a stable, productive and reproducible PCR system and cycling program for amplifying a ginseng SSR locus were obtained: 20 μ L system containing 1.0 U *Taq* DNA polymerase, 2.0 mmol·L⁻¹ Mg^{2+} , 0.2 mmol·L⁻¹ dNTPs, 0.3 μ mol·L⁻¹ SSR primer, 60 ng· μ L⁻¹ DNA template, performed with a program of 94°C for 5 min, 94°C for 30 s, annealing at 56.3°C for 30 s, 72°C for 1 min, 37 cycles, finishing at 72°C for 7 min, and storing at 4°C.

Keywords: *Panax ginseng* C.A.Meyer; Orthogonal design; SSR-PCR

Introduction

Ginseng (*Panax ginseng* C.A.Meyer) is a valuable medicinal plant and an important economic crop. China, Korea, Japan and Russia are the four major ginseng producers in the world. However, because of human excessively exploitation for ginseng resources, the wild ginseng is reducing sharply, which makes it on the verge of extinction. Ginseng has a long planting history in China. These planted ginseng plants retain all the major quantifiability of the wild plants.

In recent years, genetic variation of ginseng has been extensively studied by various methods, such as DALP, RAPD and AFLP (Wang 2004; Jiang 1998; Ma 2000). Thereinto, breed identification of ginseng has been one of the most important directions.

Simple sequence repeat (SSR) (Tautz 1989), also known as microsatellites DNA (Litt & Luty 1989), had been one of the most powerful genetic markers in biology. They were common, readily identified DNA features consisting of short (1–6 bp), tandemly repeated sequences, widely and ubiquitously distributed throughout eukaryotic genomes (Tóth 2000). In particular, Katti *et al.* (Katti 2001) have recently published a very thorough description of simple sequence repeats in eukaryotic genome sequences. As the fourth generation of molecular markers technology, SSR was phenotype marker, with highly polymorphic, few DNA template, good stabilization and repetition and manipulation. SSR was evenly distributed all over the genome (Zhou 2005). At present, SSR was widely used in heredity multi-

plicity research, such as wheat, maize, soybean, and rice (Zhang 2002; Zhong 2006; Wang 2002; Li 2005).

SSR-PCR amplification system is important to identifying molecular markers in genetic polymorphism analysis. It relates to DNA amplification efficiency, specificity and reproducibility, influencing the statistics and analysis on bands (Dieffenbach & Dveksler 2002). PCR system might be affected by many factors. Moreover, few studies on establishment of SSR-PCR system for ginseng were reported. This experiment based on other crop SSR reaction system to optimize SSR-PCR system of ginseng by an orthogonal design $L_{16}(4^5)$ (four levels of five factors: *Taq* DNA polymerase, Mg^{2+} , DNA template, dNTP and primer) (Qiao 2004).

Materials and methods

Materials

Two years old fresh ginseng leaves came from the Institute of Special Economic Animal and Plant Sciences, CAAS. DNA was extracted using the CTAB method (Scott O R 1985), quantified by using Beckman DU640 Nucleic Acid and Protein Analyzer, and diluted to 60 ng· μ L⁻¹ with ddH₂O. *Taq* DNA polymerase, Mg^{2+} and dNTP were purchased from MBI Fermentas Corporation. Primers StkVa134 (Forward primers 5'-GGATTTCAGGGCTAAGATTGCTA-3' and Reverse primers 5'-TTTGTGGAGATCAAACCATCTC-3') and RgVamu111 (Forward primers 5'-ACCAGAGAGTGGTGGGACAC-3' and Reverse primers 5'-CCTTTTATCTTGTAATACTGCCTGA-3') were synthesized by Shanghai Sangon Biological Engineering Technology Corporation. Molecular Marker DL2000 was provided by TaKaRa Corporation.

Factors and levels of PCR and $L_{16}(4^5)$ orthogonal design

To obtain the optimal conditions of five factors in PCR, SSR-PCR amplification system on ginseng was optimized by the orthogonal design $L_{16}(4^5)$ in four levels of five factors (*Taq* DNA polymerase, Mg^{2+} , DNA template, dNTP and primer), respectively (Table 1). $L_{16}(4^5)$ orthogonal design for the factors and levels of PCR (Yuan 2000) is shown in Table 2. Each reaction was repeated twice.

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The PCR amplification was programmed in a PTC-200 Thermal Cycler (MJ Co. USA), under the following conditions: 94°C for 5 min, 94°C for 30 s, annealing at 57°C for 30 s, 72°C for 1 min, 37 cycles, finishing at 72°C for 7 min, storing at 4°C. Reaction products were isolated using 3% agarose gel containing 0.5 $\mu\text{g}\cdot\text{mL}^{-1}$ EB. The electrophoresis result was recorded using Bio Imagine System of Gene (Genius Co.) and the statistical analysis was performed by software SPSS 11.0.

Table 1. Factors and levels of PCR

Factors	Levels (Final concentration)			
	1	2	3	4
<i>Taq</i> DNA polymerase ($\text{U}\cdot(20\cdot\mu\text{L})^{-1}$)	0.5	1.0	1.5	2.0
Mg^{2+} ($\text{mmol}\cdot\text{L}^{-1}$)	1.5	2.0	2.5	3.0
DNA template ($\text{ng}\cdot(20\cdot\mu\text{L})^{-1}$)	30.0	60.0	90.0	120.0
dNTP ($\text{mmol}\cdot\text{L}^{-1}$)	0.1	0.15	0.2	0.25
Primer ($\mu\text{mol}\cdot\text{L}^{-1}$)	0.1	0.2	0.3	0.4

Table 2. $L_{16}(4^5)$ orthogonal design for the factors and levels of PCR

No.	<i>Taq</i> DNA polymerase ($\text{U}\cdot(20\cdot\mu\text{L})^{-1}$)	Mg^{2+} ($\text{mmol}\cdot\text{L}^{-1}$)	DNA template DNA ($\text{ng}\cdot(20\cdot\mu\text{L})^{-1}$)	dNTP ($\text{mmol}\cdot\text{L}^{-1}$)	Primer ($\mu\text{mol}\cdot\text{L}^{-1}$)
1	0.5	1.5	30	0.1	0.1
2	0.5	2.0	60	0.15	0.2
3	0.5	2.5	90	0.2	0.3
4	0.5	3.0	120	0.25	0.4
5	1.0	1.5	60	0.2	0.4
6	1.0	2.0	30	0.25	0.3
7	1.0	2.5	120	0.1	0.2
8	1.0	3.0	90	0.15	0.1
9	1.5	1.5	90	0.25	0.2
10	1.5	2.0	120	0.2	0.1
11	1.5	2.5	30	0.15	0.4
12	1.5	3.0	60	0.1	0.3
13	2.0	1.5	120	0.15	0.3
14	2.0	2.0	90	0.1	0.4
15	2.0	2.5	60	0.25	0.1
16	2.0	3.0	30	0.2	0.2

Optimization of annealing temperature

Primer StkVa134 was amplified with gradient annealing temperature without changing other reaction conditions: 52°C, 52.3°C, 52.9°C, 53.7°C, 54.8°C, 56.3°C, 58.0°C, 59.4°C, 60.5°C, 61.3°C, 61.8°C, and 62°C. The reaction products were separately detected in 8% polyacrylamide gel electrophoresis, 3% agarose gel electrophoresis, and 8% polyacrylamide gel electrophoresis with silver staining.

Results

Scores of amplification results

Results of PCR performed according to the design in Table 2 are shown in Fig. 1. Each reaction was repeated twice. The results were scored from 16 to 1 (He 1998). All of the reaction had a high reproducibility. The first amplification using primer pair StkVa134 was scored as follow: 1, 4, 3, 2, 13, 11, 6, 5, 8, 12, 14, 10, 15, 16, 7, 9, and the repeated amplification was scored as 3, 1, 2, 4, 11, 10, 7, 5, 13, 12, 9, 15, 14, 16, 8, 6.

Variance analysis of each factor

The result indicates that all factors also affect the efficiency of PCR, with an order of high-to-low importance: *Taq* DNA polymerase, primer, Mg^{2+} , dNTP, and DNA template (Table 3).

Table 3. Variance analysis for the factors of PCR

Source	DF	SS	MS	<i>F</i>
<i>Taq</i> DNA polymerase	3	432.250	144.083	41.915**
Mg^{2+}	3	73.000	24.333	7.079*
DNA template	3	5.250	1.750	0.509
dNTP	3	7.750	2.583	0.752
Primer	3	106.750	35.583	10.352**
Error	16	55.000	3.438	
Corrected Total	31	680.000		

Note: ** significant difference at the 0.01 level; * significant difference at the 0.05 level



Fig. 1 Result of PCR amplification

Note: M=Marker DL2000; 1-16: Treatment No. 1-16, treatments as showed in Table 2

Effects of each factor on PCR

Taq DNA polymerase

Taq DNA polymerase has an impact on the reproducibility of PCR with concentration changes. From the level of 0.5 U to 1.5

U, the reaction score exhibits an increasing trend, and then declines as the concentration more than 1.5 U (Fig. 2). When the concentration of *Taq* DNA polymerase is lower, PCR products are little and the brightness is weaker. When the concentration of *Taq* DNA polymerase is 1.0 U level, the reaction is perfect, with clear band and moderate background.

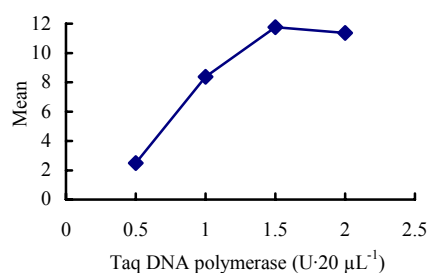


Fig. 2 Relationship between concentration of *Taq* DNA polymerase and the reaction scores

Mg²⁺ concentration

As shown in Fig. 3, with the increasing of Mg^{2+} concentration from 1.5 mmol·L⁻¹ to 2.0 mmol·L⁻¹, the result of reaction slightly increases, then starts to decline with the increasing Mg^{2+} concentrations. According to formerly experiences and this experimental gradient results, 2.0 mmol·L⁻¹ Mg^{2+} is the optimal concentration for PCR system of ginseng.

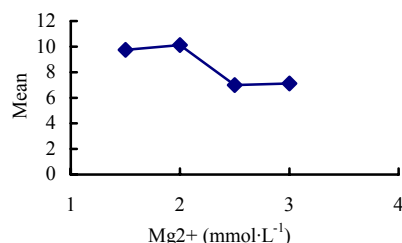


Fig. 3 Relationship between Mg^{2+} concentration and the reaction scores

Template DNA

The concentration (30–120 ng) of template DNA is tested in a total reaction volume of 20 μL. Results show that the concentration in this rang hasn't significant effect on the reproducibility and productivity, and 60 ng is recommended (Fig. 4).

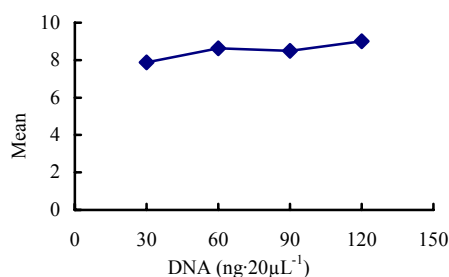


Fig. 4 Relationship between DNA template concentration and the reaction scores

dNTP

From Fig. 5, we can see that there is a slight decrease with the increase of dNTP concentration. Thus, concentration of dNTP less than 0.2 mmol·L⁻¹ is recommended.

Primer

Based on Fig. 6, with primer concentration increasing, the production increases without reducing reproducibility. But the

higher primer concentration results in higher yield of primer dimer. Considering economy and primer dimer, 0.3 μmol·L⁻¹ is recommended.

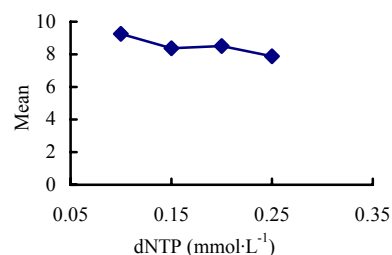


Fig. 5 Relationship between dNTP concentration and the reaction scores

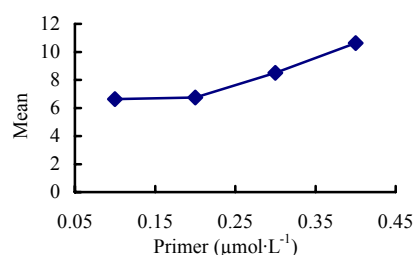
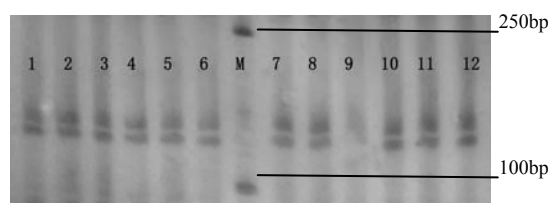


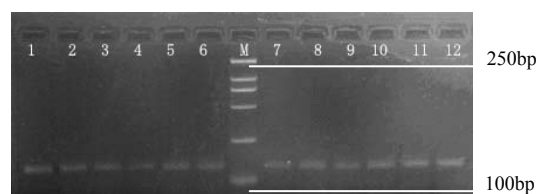
Fig. 6 Relationship between primer concentration and the reaction scores

Annealing temperature and electrophoresis results of PCR products

Annealing temperature was tested between 52°C and 62°C in optimized PCR system using primer StkVa134 as above. Electrophoresis showed non-specific band on either agarose gel or polyacrylamide gel when annealing temperature was below 52.9°C. Instead, specific band was observed between 53.7°C and 59.4°C, with the highest productivity at 57°C. This suggested that the annealing temperature of primer should be optimized individually.



a: polyacrylamide gel electrophoresis



b: agarose gel electrophoresis

Fig. 7 The impact of annealing temperature on SSR amplification

Note: Lane 1-12 are corresponding to 52°C, 52.3°C, 52.9°C, 53.7°C, 54.8°C, 56.3°C, 58.0°C, 59.4°C, 60.5°C, 61.3°C, 61.8°C, 62°C; M: Marker DL2000

Discussion

At present, SSR technique has been widely applied in gene orientation, inheritance map and polymorphism analysis proverbially. PCR is a very important process during the SSR amplification system. Different SSR-PCR amplification system needs different reaction conditions, so it is crucial that aiming at different species to establish an optimal PCR system. In former study, random thoroughly experiment was conducted to study the best combination among factors (Xia 2004). It is also reported the PCR system was established using combination of grads enactment and single factor (Zhang 2005). In this study, a SSR-PCR amplification system has been optimized with the orthogonal design $L_{16}(4^5)$ using ginseng DNA as template. Orthogonal design provides a framework to test multiple factor at multiple levels and proposes a most optimized factor combination. Additionally, scoring of PCR results played a key role in assessing the impact of factors and their levels as proposed by Qiao (2004). Scoring is a process making a result digitalized. Therefore, it depends on the experience of the operator.

In this study, all experiments were conducted under a similar lab condition and using the same set of instruments and reagents. This reduced the errors caused by system and environment. We also scored each result for twice or more, so that the error caused by person was minimized. Therefore, a stable, productive and reproducible PCR system and cycling program for amplifying a ginseng SSR locus were obtained: 20 μL system containing 1.0 U *Taq* DNA polymerase, 2.0 $\text{mmol}\cdot\text{L}^{-1}$ Mg^{2+} , 0.2 $\text{mmol}\cdot\text{L}^{-1}$ dNTPs, 0.3 $\mu\text{mol}\cdot\text{L}^{-1}$ SSR primer, 60 $\text{ng}\cdot\mu\text{L}^{-1}$ DNA template, performed with a program of 94°C for 5 min, 94°C for 30 s, annealing at 56.3°C for 30 s, 72°C for 1 min, 37 cycles, finishing at 72°C for 7 min, and storing at 4°C.

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